Technische Universiteit
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iGEM TU/e 2016
Biomedical Engineering

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Vector Linearization

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1 Vector Linearization through PCR

Estimated bench time: 45 minutes
Estimated total time: 5-7 hours (depends on the vector)
Purpose: Preparing a linear vector which can be used in the Gibson Assembly reaction.

1.1 Materials

- Autoclaved H_2O
- Bucket with ice
- Pair of primers which yield the necessary overlaps for the insert
- PCR tubes
- Pipettes and tips
- Q5 High-Fidelity 2X Master Mix
- PCR machine
- Vector
- Vortex

1.2 Setup & Protocol

- Thaw the Q5-HF 2X Master Mix on ice. If the Master Mix contains a pellet, briefly vortex the tube until the pellet disappears.
- Set up a PCR with the following reaction components for the vector to be amplified. Add the Q5-HF 2X Master Mix lastly! Quickly transfer the PCR tube to the PCR machine adding the polymerase:

Component	**Stock Concentration**	**Final Concentration**	**Volume (ul)**
Plasmid	1 ng/ul	1 ng	2
Forward primer	10 uM	0.5 uM	2.5
Reverse primer	10 uM	0.5 uM	2.5
Q5 High-Fidelity 2X Master Mix	2X	1X	25
H_2O			18
Total			50

- Run the following PCR program:

Step	**Temperature (°C)**	**Time (sec.)**	**Cycles**
Initial Denaturation	98	120	1
Denaturation ***Annealing**** ***Extension****	98 72[1] 72	15 20 30/kb → 162	35
Final extension*	72	120	1
Hold	4		

*Differs per PCR dependent on Tm of primers!

[1] The annealing temperature can be calculated for the set of primers using New England Biolabs Tm calculator.

2 DpnI digestion

Estimated bench time: 5 minutes plus 1 minute per sample
Estimated total time: 1.5 hours
Purpose: Digestion of the template vector from the PCR product mixture. This will remove the number of background colonies which do not carry the desired insert after Gibson Assembly.

2.1 Materials

- 10X CutSmart buffer from New England Biolabs
- Bucket with ice
- DpnI restriction enzyme
- PCR product
- PCR machine

2.2 Setup & Protocol

- Thaw the 10X CutSmart buffer at room temperature.
- Thaw the DpnI restriction enzyme on ice. Setup the following reaction:

Component	**Stock concentration**	**Final concentration**	**Volume**
PCR product			50
10X CutSmart buffer	10X	1X	5.7
DpnI	20U/ul	20U	1
Total	2X	1X	56.7

- Digest the vector for 1 hour at 37 °C. Heat inactivate DpnI for 20 minutes at 80 °

3 PCR Purification

Estimated bench time: 45 minutes
Estimated total time: 45 minutes
Purpose: If the PCR product is less than 90% pure, large volumes of unpurified PCR product could significantly inhibit the Gibson Assembly. PCR purification may be performed to increase the efficiency.

For more information, see our general PCR Purification protocol.

4 NanoDrop

Estimated bench time: 5 minutes start-up and 2 minutes per sample
Estimated total time: 5 minutes start-up and 2 minutes per sample
Purpose: Measuring the concentration of the PCR product which is necessary to set up the Gibson Assembly reaction.

For more information, see our general NanoDrop protocol.

5 Gel Electrophoresis

Estimated bench time: 40 minutes
Estimated total time: 1.5 hours
Purpose: Agarose gel electrophoresis may be used to verify the purity of your PCR product. If the product is pure, a single bond will show up during the gel electrophoresis.

For more information, see our general Gel Electrophoresis protocol.

6 References & Acknowledgements

This protocol was based on the information provided by the 2015 iGEM team of the TU/Eindhoven, New England Biolabs NEBuilder HiFi DNA Assembly Cloning Kit manual and on Integrated DNA Technologies' g Blocks Gene Fragments Cloning Protocols.